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Hypothermic Oxygenated Perfusion (HOPE) downregulates the immune response in a rat model of liver transplantation

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Abstract: OBJECTIVE: To evaluate the impact of a novel oxygenated perfusion approach on rejection after orthotopic liver transplantation (OLT). BACKGROUND: Hypothermic oxygenated perfusion (HOPE) was designed to prevent graft failure after OLT. One of the mechanisms is downregulation of Kupffer cells (in situ macrophages). We, therefore, designed experiments to test the effects of HOPE on the immune response in an allogeneic rodent model of nonarterialized OLT. METHODS: Livers from Lewis rats were transplanted into Brown Norway rats to induce liver rejection in untreated recipients within 4 weeks. Next, Brown Norway recipients were treated with tacrolimus (1 mg/kg), whereas in a third group, liver grafts from Lewis rats underwent HOPE or deoxygenated machine perfusion for 1 hour before implantation, but recipients received no immunosuppression. In a last step, low-dose tacrolimus treatment (0.3 mg/kg) was assessed with and without HOPE. RESULTS: Allogeneic OLT without immunosuppression led to death within 3 weeks after nonarterialized OLT due to severe acute rejection. Full-dose tacrolimus prevented rejection, whereas low-dose tacrolimus led to graft fibrosis within 4 weeks. HOPE treatment without immunosuppression also protected from lethal rejection. The combination of low-dose tacrolimus and 1-hour HOPE resulted in 100% survival within 4 weeks without any signs of rejection. CONCLUSIONS: We demonstrate that allograft treatment by HOPE not only protects against preservation injury but also impressively downregulates the immune system, blunting the alloimmune response. Therefore, HOPE may offer many beneficial effects, not only to rescue marginal grafts but also by preventing rejection and the need for immunosuppression.

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Hypothermic Oxygenated Perfusion (HOPE) Downregulates the Immune Response in a Rat Model of Liver Transplantation

Andrea Schlegel, MD, Philipp Kron, MD,* Rolf Graf, PhD,* Pierre-Alain Clavien, MD, PhD,*† and Philipp Dutkowski, MD**

Objective: To evaluate the impact of a novel oxygenated perfusion approach on rejection after orthotopic liver transplantation (OLT).

Background: *H*ypothermic *o*xygenated *per*fusion (HOPE) was designed to prevent graft failure after OLT. One of the mechanisms is downregulation of Kupffer cells (in situ macrophages). We, therefore, designed experiments to test the effects of HOPE on the immune response in an allogeneic rodent model of nonarterialized OLT.

Methods: Livers from Lewis rats were transplanted into Brown Norway rats to induce liver rejection in untreated recipients within 4 weeks. Next, Brown Norway recipients were treated with tacrolimus (1 mg/kg), whereas in a third group, liver grafts from Lewis rats underwent HOPE or deoxygenated machine perfusion for 1 hour before implantation, but recipients received no immunosuppression. In a last step, low-dose tacrolimus treatment (0.3 mg/kg) was assessed with and without HOPE.

Results: Allogeneic OLT without immunosuppression led to death within 3 weeks after nonarterialized OLT due to severe acute rejection. Full-dose tacrolimus prevented rejection, whereas low-dose tacrolimus led to graft fibrosis within 4 weeks. HOPE treatment without immunosuppression also protected from lethal rejection. The combination of low-dose tacrolimus and 1-hour HOPE resulted in 100% survival within 4 weeks without any signs of rejection.

Conclusions: We demonstrate that allograft treatment by HOPE not only protects against preservation injury but also impressively downregulates the immune system, blunting the alloimmune response. Therefore, HOPE may offer many beneficial effects, not only to rescue marginal grafts but also by preventing rejection and the need for immunosuppression.

Keywords: HOPE, immune response, liver transplantation, oxygen, rejection

(*Ann Surg* 2014;260:931–938)

Orthotopic liver transplantation (OLT) offers a unique chance to treat patients with end-stage liver disease. The persistent need for lifelong immunosuppressive treatment, however, is a major shortcoming in terms of several serious side effects including infectious complications,[1] long-term nephrotoxicity,[2] and primary tumor recurrence[3] or secondary tumor growth.[4] For these reasons, novel strategies decreasing the need of immunosuppressive treatment after transplantation seem highly attractive.

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Recently, we have presented the worldwide first results of a newly developed machine liver perfusion approach for human liver grafts, donated after cardiac death.[5] The perfusion technique consisted of a short-term hypothermic oxygenated perfusion (HOPE) after conventional organ procurement and cold storage and was shown to protect effectively from ischemia-reperfusion (I/R) injury despite long donor warm ischemia times.[6–8] We have also reported, in several experimental studies, that such effects of HOPE depend on oxygenation of the perfusate[6] with decreased downstream activation of numerous inflammatory pathways.[6,7] On the basis of these findings, the aim of the current study was to analyze effects of HOPE treatment on the immune response in a model of acute rejection, using incompatible rat strains for OLT.[9] We compared allogeneic liver transplants with and without immunosuppressive or HOPE treatment. In addition, to provide mechanistic insights, we added perfusion experiments with a deoxygenated perfusate. We focus on T-cell response and survival within 30 days after liver transplantation.

METHODS

Animals

Male Lewis and Brown Norway rats (250–320 g) were used for all experiments. Animals received standard laboratory diet and water according to the Swiss Animal Health Care law, and experiments were approved by the animal ethics committee. Anesthesia during liver procurement and transplantation was maintained with isoflurane.

Study Design

Transplantation of livers from Lewis rats into Brown Norway recipients has previously been shown to induce rejection.[9–12] Using this strain combination, we compared in an allogeneic liver transplant model the impact of HOPE against standard immunosuppression. We selected the following experimental groups (see Supplemental Digital Content Fig. 1, available at http://links.lww.com/SLA/A649):

I. *Syngeneic control*: Livers from Brown Norway rats were procured and implanted into Brown Norway recipients (*Syngeneic control*).
II. *Untreated group:* Livers from Lewis rats were procured and transplanted into Brown Norway recipients without any additional treatment (*Untreated).*
III. *Full immunosuppression*: Livers from Lewis rats were procured and transplanted into Brown Norway recipients, which received full immunosuppressive treatment with tacrolimus intramuscularly (1 mg/kg of bodyweight/d), starting before recipient hepatectomy *(TAC).*
IV. *Low-dose immunosuppression:* Livers from Lewis rats were procured and transplanted into Brown Norway recipients, which received a reduced immunosuppressive treatment with tacrolimus intramuscularly (0.3 mg/kg of bodyweight/d), starting before recipient hepatectomy *(low TAC).*
V. *HOPE group*: Livers from Lewis rats were procured, machine perfused with an oxygenated perfusate (HOPE) for 1 hour, and transplanted without any additional recipient treatment (*HOPE*).

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VI. *HNPE group*: Livers from Lewis rats were procured, machine perfused with a nitrogenated perfusate (HNPE) for 1 hour, and transplanted without any additional recipient treatment (*HNPE*).
VII. *HOPE + low-dose immunosuppression*: Livers from Lewis rats were procured, machine perfused with an oxygenated perfusate (HOPE) for 1 hour, and transplanted into Brown Norway recipients, which received a reduced immunosuppressive treatment with tacrolimus intramuscularly (0.3 mg/kg of bodyweight/d), starting before recipient hepatectomy *(HOPE + low TAC)*.

Endpoints

We analyzed hepatocyte necrosis, Kupffer cell activation, endothelial cell activation, and T-cell activation by specific staining procedures 24 hours after OLT in each group. In additional experiments, we documented plasma alanine aminotransferase (AST), bilirubin, high mobility group box-1 protein (HMGB-1), 8-hydroxy-2-deoxy guanosine (8-OHdG), interleukin (IL)-2, IL-10, and interferon gamma (IFN-γ) during 14 days after OLT. The T-cell response after 2 weeks was investigated by fluorescence-activated cell-sorting (FACS) analysis in blood samples. Additional staining procedures were performed 2 and 4 weeks after transplantation to confirm tissue remodeling into liver fibrosis and rejection. Follow-up for survival after OLT was 4 weeks.

Liver Procurement and OLT

Donor livers were freed from ligaments and flushed with 6 mL of heparinized (1 U/mL) saline at room temperature via the portal vein. Livers were excised (weight 9.7 ± 1.5 g) and placed in precooled UW solution (4°C). Afterward, non–machine-perfused livers received cuffs for the portal vein and the infrahepatic vena cava whereas livers allocated to the perfusion groups received first a stent for the portal vein and later cuffs after machine perfusion. Cold storage was approximately 30 minutes in all experimental groups. Nonarterialized liver transplantation was performed after procurement or machine perfusion according to the technique by Kamada and Calne.[13]

HOPE and HNPE

Livers from Lewis rats to be cold perfused were connected to the precooled perfusion device and perfused for 1 hour through the portal vein with a constant perfusion pressure of 3 mm Hg or less.[14] We used 50 mL of recirculating modified starch-free UW solution as perfusate.[7,8] Perfusion box and perfusate were maintained at 4°C by an open bath thermostat (Huber, Germany). In all *HOPE* experiments, the cold perfusate was actively oxygenated (po_2 >60 kPa) (*HOPE group*). In the *HNPE* group, oxygen was replaced by nitrogen (po_2 <2 kPa) (*HNPE group*).

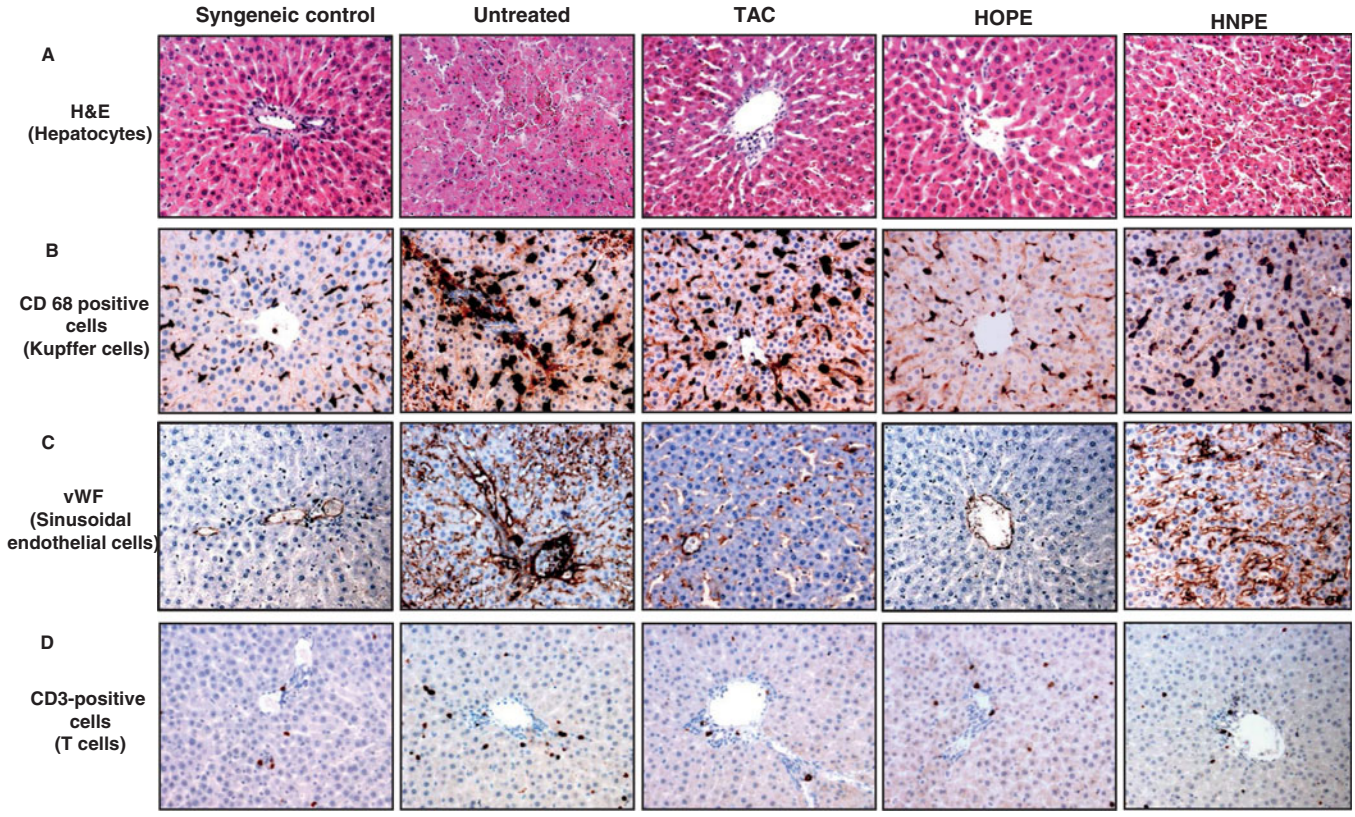

FIGURE 1. Liver injury 24 hours after OLT: allogeneic liver transplantation without immunosuppression induced hepatocyte injury and Kupffer and endothelial cell activation during the first day after reperfusion (A–C). HOPE treatment significantly protected hepatocytes from reperfusion injury (A). Macrophages and endothelial cells after HOPE also appeared less activated (B, C). Machine perfusion with a deoxygenated perfusate (HNPE) induced the same degree of injury after OLT as untreated allogeneic controls (A–C). Treatment with tacrolimus showed less effect on Kupffer cell activation compared with HOPE (B). One day after OLT, CD3-positive T cells in livers were rarely detectable in all experimental groups (D). H&E indicates hematoxylin-eosin; TAC, tacrolimus; vWF, von Willebrand factor.

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Assays

Hepatocyte injury after transplantation was measured by AST release and total bilirubin (serum multiple biochemical analyzer DRI-CHEM4000i; FUJIFILM, Japan). Oxidative damage of DNA by oxygen free radicals was detected using an 8-OHdG enzyme-linked-immunosorbent assay (Abnova, KA0444). Nuclear subcellular injury was measured by release of HMGB-1 using a specific enzyme-linked-immunosorbent assay (IBL International GmbH, ST51011). In addition, several markers indicating graft rejection were analyzed in the rat plasma after transplantation, that is, IL-2 (IBL, 87728012), IL-10 (R&D, R1000), and IFN-γ (R&D, RIF00).

FACS Analysis

After blood retrieval, 1.5 mL of heparinized full rat blood was used for this analysis. After white blood cell isolation using Ficoll and DMEM+P/S buffer, cells were incubated with various combinations of mAbs (anti-Rat CD3, PerCP-eFluor710 single staining, anti-rat CD4-PE combined with anti-rat CD25-FITC, anti-rat CD8-FITC combined with anti-rat CD28-PE), washed twice with FACS buffer, and fixed with 1% paraformaldehyde. Three-color immunofluorescence staining was analyzed using a FACS Calibur instrument (FACS diva, version 6.1.2). The lymphocytes were gated using forward and side scatter to exclude debris and dead cells. Afterward, 50,000 events were acquired in each assay for analysis.

Histological Examinations

The following staining procedures were performed after transplantation: hematoxylin-eosin staining for necrosis, Sirius red for fibrosis, CD68 staining for Kupffer cell activation, CD80/86 staining for antigen presenting cells, von Willebrand factor staining for endothelial activation, and CD3 staining for T lymphocytes. Quantification of CD68-positive cells, CD80-positive cells, von Willebrand factor–positive endothelial cells, and CD3-positive lymphocytes in liver tissue was performed in 8 random fields of 2 slides per animal, resulting in 160 quantified fields per experimental group. Acute rejection was assessed on hematoxylin-eosin staining and based on portal inflammation, bile duct inflammation, and endothelial inflammation,[15] and signs of chronic rejection based on portal inflammation, bile duct damage, obstructive arteriopathy, and graft fibrosis.[16]

Statistics

Data are presented as means ± standard deviation. Statistical analysis was performed using the nonparametric Mann-Whitney-Wilcoxon *U* test (GraphPadPrism, version 4.0; San Diego, CA).

RESULTS

Acute Rejection Without and With Immunosuppressive Therapy in an Allogeneic Rat Liver Transplant Model

In a first step, we documented the degree of liver graft injury in an allogeneic rat liver transplant model (donor livers from Lewis rats into Brown Norway recipients)[9,10] in contrast to syngeneic transplant controls. Recipients without immunosuppressive treatment developed severe graft injury within 24 hours after OLT, confirmed by nuclear

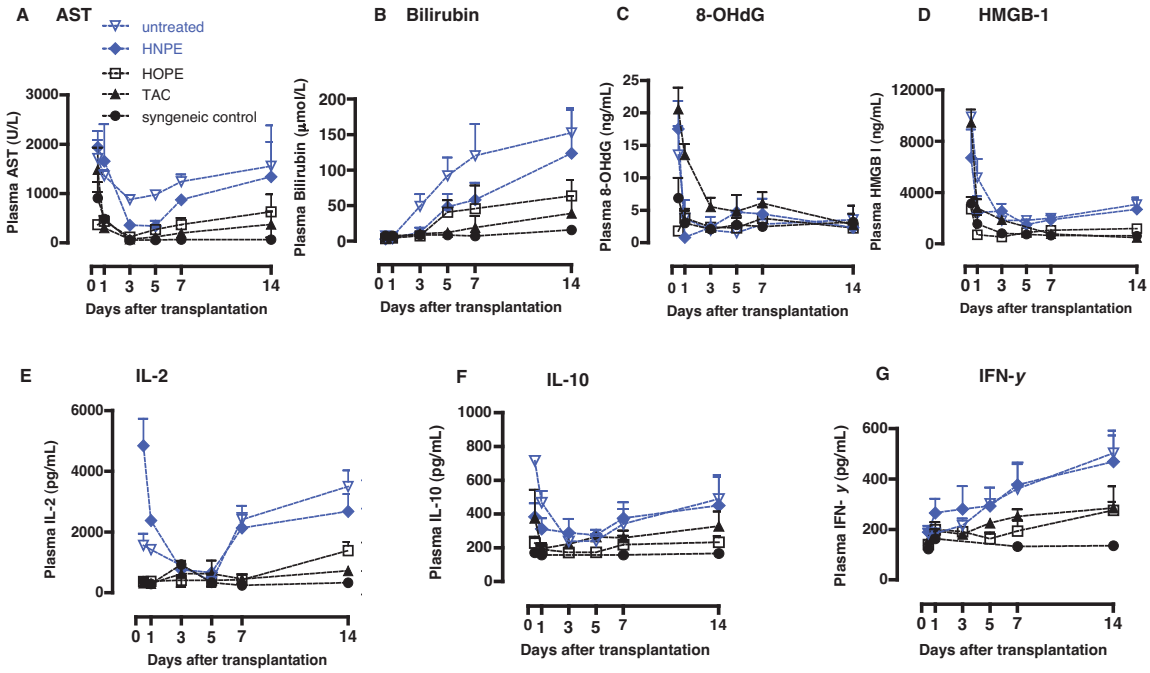

FIGURE 2. Liver injury during 2 weeks after allogeneic OLT: Recipients without immunosuppression developed elevated levels of liver enzymes (A), bilirubin (B), IL-2, IL-10 (E, F), and IFN-γ (G). Nuclear injury and oxidative stress markers (ie, HMGB-1, and 8-OHdG) increased in untreated allogeneic grafts and also in livers perfused with deoxygenated perfusate (HNPE group) (C, D). Immunosuppression (TAC) had no effect on initial DAMP molecule and reactive oxygen species release but protected from the release of rejection markers (E–G). HOPE treatment led, first, to significantly decreased reperfusion injury, as shown by less amounts of oxidized DNA (C) and HMGB-1 (D), and second, induced less IL-2, IL-10, and IFN-γ release (E–G) during 2 weeks after OLT. Machine perfusion without oxygen abrogated the effect of HOPE (A–G). TAC indicates tacrolimus.

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injury (8-OHdG and HMGB-1 release), AST release, and Kupffer and endothelial cell activation (Figs. 1, 2). Subsequently, in these animals, we observed acute rejection during the first 2 weeks after transplantation, as documented by massive T-cell infiltration (Fig. 3), along with persistent high blood levels of AST, bilirubin, IL-2, IL-10, and IFN-γ (Fig. 2). In addition, Sirius red staining in liver samples after 2 weeks revealed severe graft fibrosis (Fig. 4). Such degree of injury was lethal in all untreated recipients during 18 days after allogeneic OLT (Fig. 3) despite choosing experimental conditions with minimal exposure of liver grafts to cold and warm ischemia (see Supplementary Fig. 1, available at http://links.lww.com/SLA/A649).

In a next step, we treated recipients before graft implantation with tacrolimus (1 mg/kg of body weight/d),[9] resulting in a trough level of 8 to 10 ng/l, according to earlier studies.[9] Such degree of immunosuppression protected significantly from endothelial cell activation 24 hours after OLT (Fig. 1). In addition, within the following 2 and 4 weeks, infiltration of T cells in liver grafts remained low (Fig. 3). These findings were paralleled by the decreased amount of circulating activated T cells (Fig. 3) and low cytokine levels in blood (Fig. 2). Recipient survival increased to 80% within 4 weeks (6/8) (Fig. 3).

Finally, we reduced immunosuppressive therapy to one third of the daily dose (0.3 mg/kg of body weight/d) resulting in a trough level of 3 to 4 ng/l. With this low-dose tacrolimus therapy, recipients survived (6/8) but developed liver fibrosis within 4 weeks after OLT (Fig. 5).

In summary, allogeneic nonarterialized rat liver transplantation without immunosuppression led to severe acute rejection, liver fibrosis, and animal death within 3 weeks. Lethal rejection and fibrosis within 4 weeks were prevented with adequate tacrolimus treatment of recipients, whereas low-dose tacrolimus induced graft fibrosis and chronic graft injury.

Liver Graft Protection by Short-term Application of HOPE Without Immunosuppressive Treatment

To investigate the effects of HOPE on the immune response in the same allogeneic transplant model, we perfused donor livers from Lewis rats for 1 hour and implanted perfused liver grafts in Brown Norway recipients, without any immunosuppressive treatment. As expected from previous studies,[6,8] HOPE reduced reperfusion injury within the first 24 hours after OLT, confirmed by minimal 8-OHdG, HMGB-1, and AST release and decreased activation of Kupffer and endothelial cells (Figs 1, 2). Furthermore, HOPE treatment prevented infiltration of CD3-positive T cells in liver grafts (Fig. 3) consistent with low levels of IFN-γ, IL-2, and IL-10 (Fig. 2). Two weeks after HOPE treatment and OLT, quantification of circulating activated CD3-positive T cells remained low (Fig. 3) and graft fibrosis and

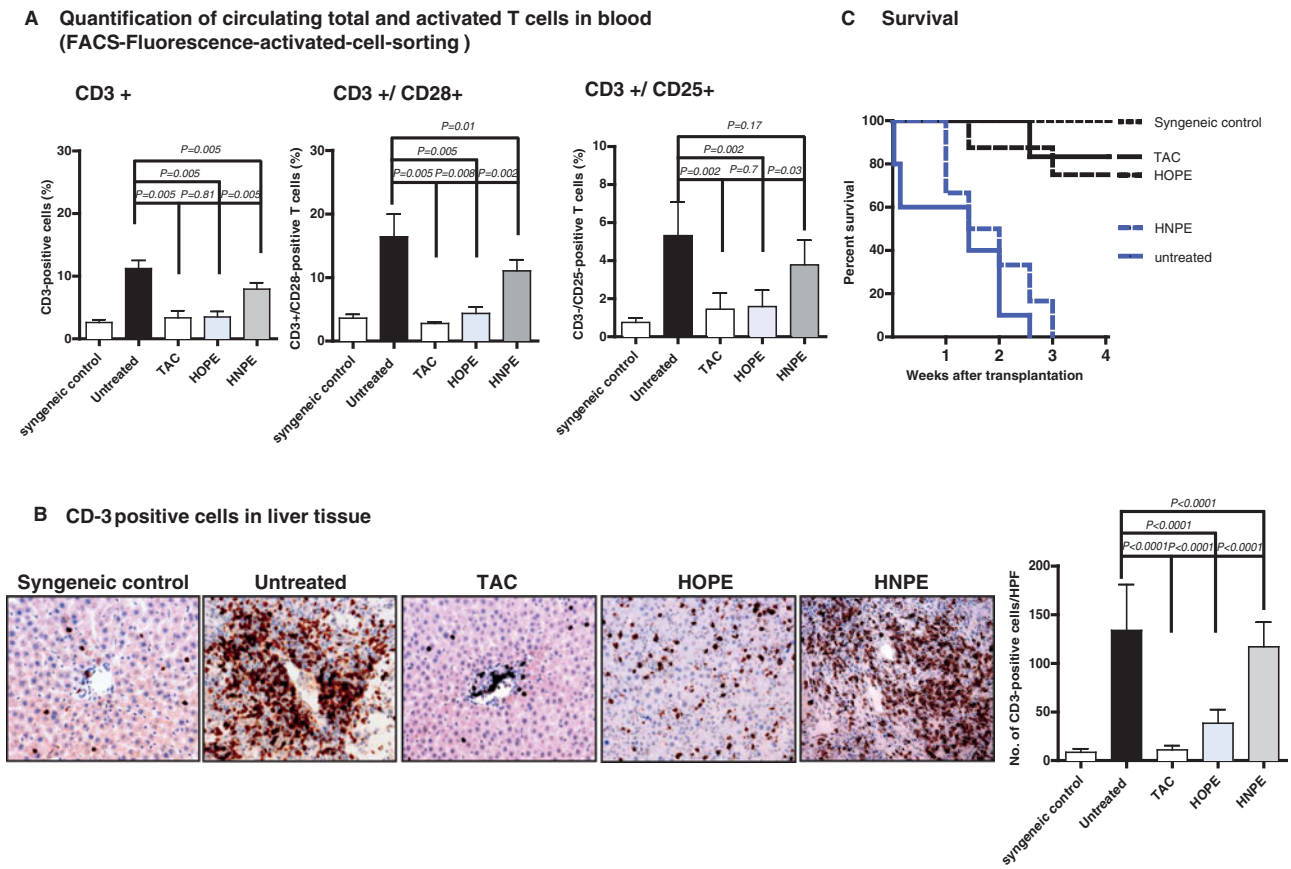

FIGURE 3. T-cell activation and survival 2 to 4 weeks after OLT: The number of circulating T cells in blood was quantified by FACS analysis (A). CD28- and CD25-positive T cells were less observed in recipients of HOPE-treated livers and also in animals with full immunosuppression (FACS analysis) (A). The number of infiltrating T cells in liver grafts was significantly reduced in HOPE- and TAC-treated animals (B). HOPE and TAC treatment increased recipient survival, whereas untreated controls died within 2 weeks after OLT (C). TAC indicates tacrolimus.

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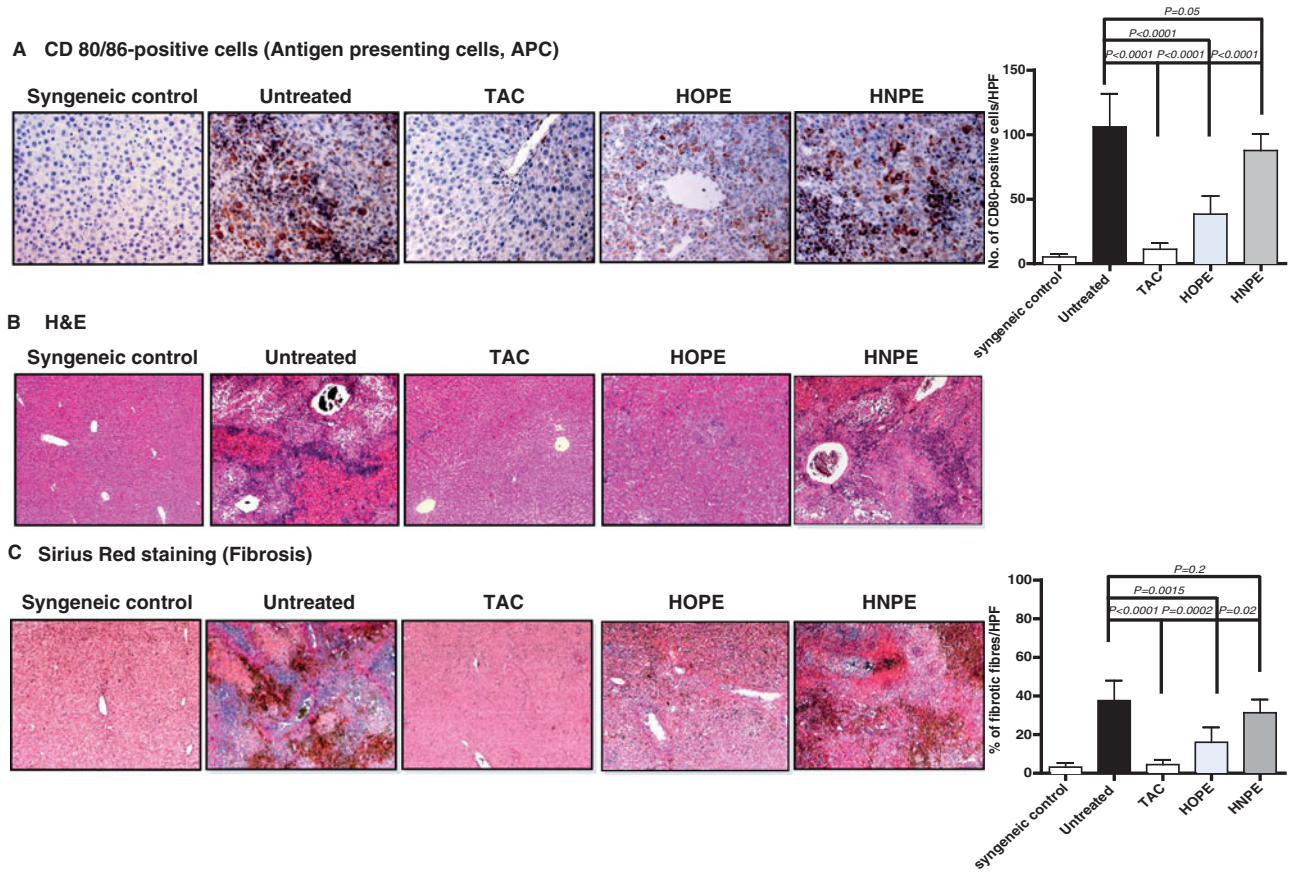

FIGURE 4. HOPE treatment led to significant reduction of CD80/86-positive cells and protected from high-grade fibrosis compared with untreated recipients and machine perfusion without oxygen (HNPE) (A–C). However, HOPE without any immunosuppression induced mild fibrosis (C) not seen under full immunosuppression (TAC) (A–C). H&E indicates hematoxylin-eosin; HPF, high power field; TAC, tacrolimus.

survival appeared significantly improved by HOPE compared with untreated liver grafts within 4 weeks after OLT (Figs. 3, 4). However, graft histology after 4 weeks disclosed increased signs of chronic injury comparable with those seen with low-dose tacrolimus treatment (Fig. 5).

On the basis of these experiments, we conclude that HOPE treatment of grafts protects from activation of the early immune response in recipients and rescued from lethal injury in an allogeneic nonarterialized liver transplant model. Yet, HOPE alone, without any further immunosuppression, failed to prevent chronic graft injury.

Immune Response After Machine Perfusion Without Oxygen

Previous results have shown that protection against I/R injury depended on the presence of oxygen in the machine perfusate during HOPE.[6] Here, we analyzed whether early immune response of the recipient was also mediated through oxygenation effects. For this purpose, we perfused liver grafts from Lewis rats with a nitrogenated perfusate (*HNPE*) at 4°C (pO_2 level <2 kPa). Livers perfused with this technique demonstrated significant higher degrees of reperfusion injury after OLT in Brown Norway recipients, confirmed by release of damage associated molecular pattern (DAMP) molecules (HMBG-1), AST, and cytokines (IL-10, IL-2, IFN-γ) and Kupffer and endothelial cell activation (Figs. 1, 2). Furthermore, machine perfusion in the absence of oxygen abrogated downstream the protective effect of HOPE on activation of T cells (Fig. 3) and led to acute liver graft rejection, fibrosis (Fig. 4), and death of all recipients within 4 weeks after OLT (Fig. 3), similarly as in untreated recipients. On the basis of this, the effect on the direct immune response by HOPE seems to be mediated by perfusate oxygen and points therefore to an initial mitochondrial protection during machine perfusion, with subsequent decreased release of reactive oxygen species and DAMP molecules.

Liver Graft Protection With Low-Dose Tacrolimus in Combination With HOPE

In a last experimental step, we combined graft treatment by HOPE and low-dose immunosuppressive therapy. With this strategy, all recipients survived for 4 weeks without signs of rejection or fibrosis in contrast to recipients under low-dose tacrolimus without HOPE (Fig. 5). We conclude that the addition of HOPE before OLT allows to reduce immunosuppressive therapy without increased risk of rejection in an allogeneic rodent transplant model.

DISCUSSION

This study showed that liver allograft treatment by an easy applicable machine perfusion approach before OLT not only is effective against reperfusion injury but also prevents activation of the immune response pathways. This was evident, first, by decreased

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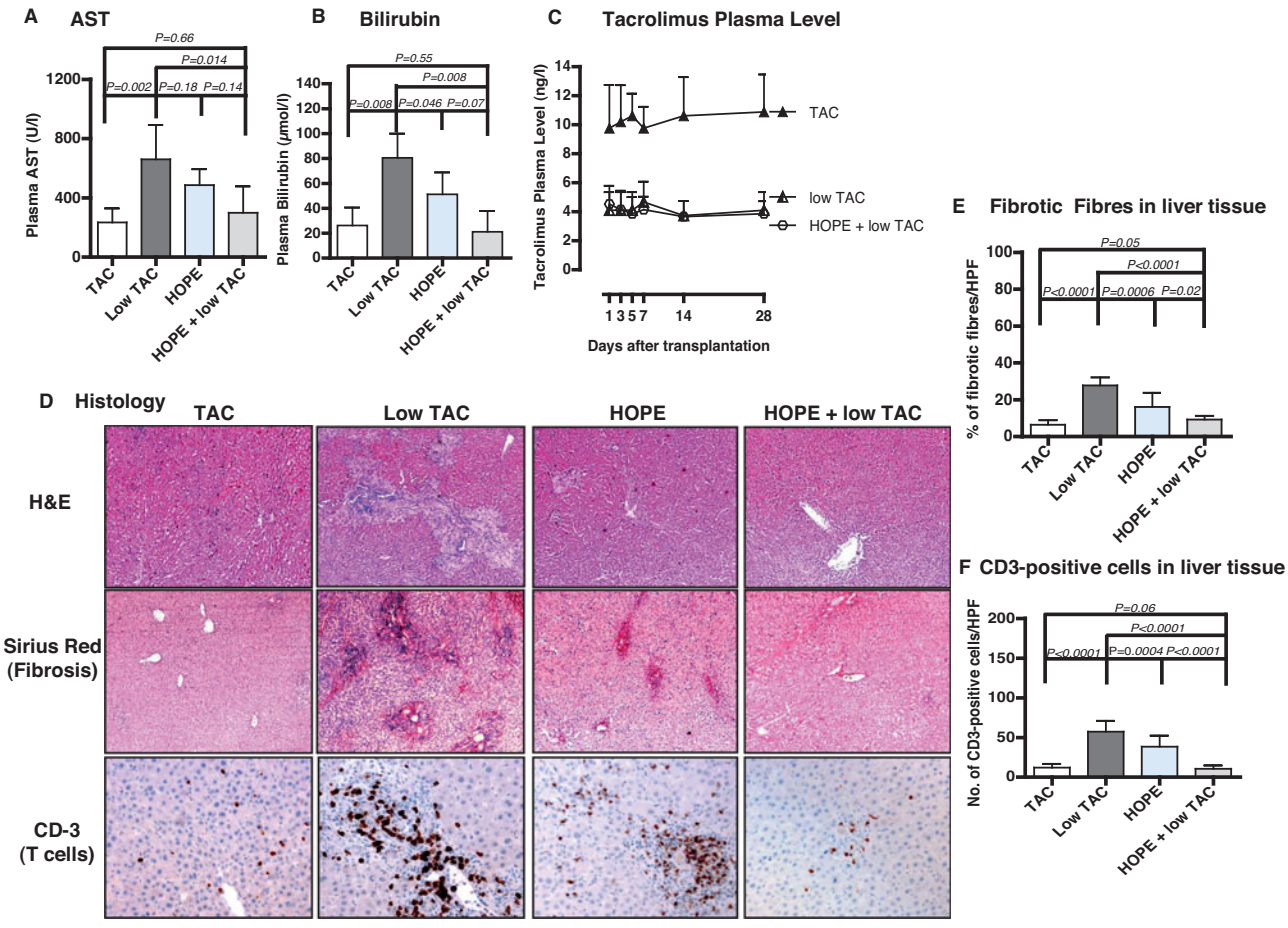

FIGURE 5. Immune response 4 weeks after OLT: Reduction of tacrolimus to levels between 4 and 5 ng/l (C) induced liver fibrosis and T-cell infiltration within 4 weeks (D). Adding HOPE before OLT prevented significantly liver injury, fibrosis, and T-cell infiltration within 4 weeks despite low-dose TAC treatment (A–F). TAC indicates tacrolimus.

Kupffer and endothelial cells activation after HOPE treatment and OLT, followed by decreased T-cell infiltration in liver grafts, and also by a decreased amount of circulating and activated T cells in blood. Of note, the initial effect of HOPE treatment on the direct immune response pathway appeared comparable with the immunosuppression conferred by tacrolimus. Furthermore, whereas a significant reduction of immunosuppressive treatment provoked graft fibrosis within 4 weeks, the addition of HOPE before OLT protected from signs of chronic graft injury.

Next, we demonstrated that the effect of HOPE against an immune response depends on oxygenation of the perfusate along with strong impact against reperfusion injury.[7] Consistently, HOPE treatment prevented from lethal graft rejection in an allogeneic transplant model whereas hypothermic perfusion using a deoxygenated perfusate failed to protect from acute rejection. It seems therefore unlikely that washout effects of immunocompetent cells during machine perfusion contribute to the decreased immune response observed in HOPE-treated livers. This important finding is consistent with previous studies, which have underlined a key role of oxygen and mitochondrial function during HOPE.[6,7,17] In parallel, end-ischemic oxygenated cold perfusion of porcine kidneys has recently been shown to reduce the innate immune response, as evaluated by HMGB-1 release and gene expression of toll like receptor-4 (TRL-4).[18]

Liver graft injury arises during the transplantation process from several sources, that is, as a result of donor brain or cardiac death, from cold storage, and from warm ischemia and reperfusion in the recipient. It is believed that hypoxia during procurement, preservation, and implantation triggers the release of reactive oxygen species in different compartments and the release of DAMP molecules, characterized by extracellular matrix fragments, nucleic acids, histones, and HMGB-1.[19,20] DAMP molecules bind to Kupffer cells, dendritic cells, leukocytes, and endothelial cells by numerous toll like receptors (TRL-2, TLR-3, TLR-4. TLR-7, TLR-9) and receptor for advanced glycation end products (RAGE).[20–24] Recognition of DAMP molecules through these receptors activate both donor- and recipient-derived dendritic cells, besides activation by other factors such as complement and lymphocytes.[14,25–27] It has recently been suggested, therefore, that prevention of an initial oxidative stress and DAMP molecule release in donors and recipients could be a key option for subsequent modulation of immune and inflammatory responses.[28] However, available free radical scavengers or other pharmacological approaches have failed in clinical practice, due to their low activity at the time and site of graft injury.[29] In contrast, hypothermic oxygenated machine perfusion before implantation, initially developed to rescue marginal liver grafts,[5] offers a unique chance to impact on the main source of intracellular oxidative stress due to changes in the mitochondrial redox state[6] before implantation. On the basis of this,

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such machine perfusion technique potentially impacts on donor immunogenic cells before any exposure to recipient cells. Accordingly, we show here, for the first time, in a rodent liver transplant model that 1-hour perfusion with a cold oxygenated perfusate before OLT was highly efficient in preventing lethal rejection, suggesting a direct link between early reperfusion injury after organ transplantation and the initiation of the immune response.[30]

Machine perfusion strategies may, therefore, not only improve initial organ function by decreasing reperfusion injury but also reduce immune response by less activation of tissue-resident dendritic cells.

We opted in this study for a nonarterialized allogeneic rat OLT model for 2 reasons. First, previous studies in rats have shown consistently that a nonarterialized rat OLT model was highly suitable for rejection studies within 4 weeks after OLT.[9,11,31,32] Second, additional hepatic artery reconstruction prolonged survival but finally failed to prevent chronic rejection and graft dysfunction.[31,32]

Translation of our results to the human situation may require longer follow-up and experiments in large animals. Further investigations should also include nonstandard liver grafts, that is, extended criteria grafts or liver grafts donated after cardiac death, as we choose in this study an experimental model without previous exposure to warm or cold ischemia.

CONCLUSIONS

HOPE-treated liver grafts may tolerate a significant lower immunosuppressive treatment. If confirmed in the clinic, the use of HOPE may open the door for regimens with significant low or delayed calcineurin inhibitors without a risk of graft rejection, but less toxic effects in high MELD recipients or donated after cardiac death liver transplants.[33,34] In addition, underlying recipient diseases that require minimal immunosuppressive treatment, that is, recurrent hepatitis C,[35] or advanced hepatic tumors[36] could benefit from this new strategy.

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DISCUSSANTS

J. Lerut (Brussels, Belgium):

Thank you very much for your elegant presentation and nice article on the potential immunologic benefit of hypothermic oxygenated machine perfusion in liver transplantation. This work is a

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good example of a well-conducted experimental work in the field of small animal liver transplantation, going 7 steps further from a syngeneic control group to a HOPE-tacrolimus group. Scientific work on the minimization of immunosuppression is especially welcomed in an era of extended criteria donors and a shift of interest within the transplant community to long-term survival.

In the "low-dose tacrolimus and HOPE" group, acute rejection and fibrosis were markers of chronic rejection and were eliminated in a model on nonarterialized liver transplantation.

In summary, all your findings come down to a well-known relationship between the degree of the I/R injury and the incidence of immunologic events, such as rejection. This link was a substantial part of the research, conducted by Prof Tilney, from Boston. As HOPE, through the impact on the mitochondrial function, downregulates the I/R injury and the innate immune response, it was logical to also search for a possible downregulation of adaptive immunologic events in your experimental setting.

You concluded that the combination of HOPE and low-dose tacrolimus eliminated rejection and fibrosis. It must, however, be said that it is nowadays possible, in clinical practice, to obtain excellent (immunologic) outcome under minimal immunosuppression. It could also be possible that the unfavorable immunologic events you documented in the non-tacrolimus groups were reinforced by the nonarterialized model of liver transplantation. So, a question arises, pertaining to the real impact of HOPE on your observations. Therefore, I have 2 questions in relation to this.

The first relates to the use of a nonarterialized rat model of liver transplantation. Indeed, the Tokyo Shimizu group showed that T-cell infiltration and immunoglobulin deposition, in sync with antibody-mediated rejection, are much more pronounced in the nonarterialized liver transplantation model. So, in the arterialized model, the effect of HOPE could be less pronounced. Do you intend to add an arterialized model of liver transplantation to your already extensive number of models?

My second question, which is of great significance, relates to a more in-depth explanation of the underlying immunologic mechanisms of your findings. HOPE alone postpones the rejection and transforms this event into a chronic add-on; even adding small doses of tacrolimus to HOPE may inhibit the B-cell response. It is, thus, necessary to observe the production of anti-HLA antibodies to confirm the immunologic protective effect of HOPE. Indeed, accelerated fibrosis may be linked to antibody production, with the following activation of the complement cascade and innate immunity (macrophages, monocytes, etc). Did you have the opportunity to observe the immune B response in your research, and if so, what was the result? This information is absolutely imperative, before one can claim that HOPE alone is able to downregulate the immune response of the liver recipient.

Response From P. Dutkowski (Zurich, Switzerland):

Thank you very much Prof Lerut for these kind comments and good questions. I would like to return to your first point, regarding an arterialized transplant model. We chose, on purpose, a nonarterialized rat liver transplantation, because, as you also mentioned, rejection is known to occur to a higher degree without arterialization. To test the maximum effect of HOPE, we, therefore, opted for a nonarterialized transplantation in this study and showed a strong effect.

The second question focuses on the effect of HOPE on the indirect immune pathway. At this stage, we cannot say something about the effect of HOPE on B-cell response and downstream mediators. This issue should be addressed by further studies.

DISCUSSANTS

M. Krawczyk (Warsaw, Poland):

Professor Dutkowski, I appreciated your article very much. Your excellent study is very important for the future of liver transplantation, as the shortage of donors is a problem for most transplant centers.

Here are my 2 questions:

First, you say that for these types of liver recipients, you would recommend the use of hypothermic oxygenated machine perfusion. Do you mean that it would be possible to use it for urgent recipients? If we have an urgent recipient, at the time of transplantation, this is really crucial. We know that the use of HOPE is time-consuming. Do you share my opinion that a benefit of HOPE has a higher effect than the time of the transplantation?

Second, I would like to ask you, whether HOPE could be used for blood group incompatibility in liver transplantation. From personal experience, I know that the number of acute rejections, in this group of patients, is higher than in blood group compatibilities in liver transplantation.

Once again, congratulations on your excellent presentation. I will be pleased to hear the answers to my 2 questions.

Response From P. Dutkowski (Zurich, Switzerland):

Thank you very much, Prof Krawczyk, for these good questions. In fact, applying HOPE is possible without losing time, as the oxygenated perfusion is done during recipient hepatectomy, which makes this method very simple.

The question, regarding blood group incompatibility, is difficult to address at the moment, because our study is the first to suggest an effect on the immune response, by a simple ex vivo graft treatment, before transplantation. Whether this technique is strong enough to avoid rejection in incompatible livers requires further investigation.

DISCUSSANTS

R. Adam (Villejuif, France):

I congratulate you and your team for acquiring further evidence for the advantage of machine perfusion on the liver.

I have one, simple question: Do you think that the minimization of ischemic injury to the liver directly influenced this type of effect on the minimization of allograph rejection, or is this a completely independent factor?

Second, to demonstrate that oxygen is key in the minimization of the graph rejection, have you done or do you plan to do an experiment with HOPE, without oxygen?

Response From P. Dutkowski (Zurich, Switzerland):

Thank you very much, Prof Adam, for your kind comments. Let me begin with your last question. We included experiments with deoxygenated perfusion, showing that the effect on the immune response was dependent on oxygen in the perfusate, consistent with earlier studies on reperfusion injury.

Indeed, as we minimized liver ischemia in this model of the allogeneic rat liver transplantation, we would expect a higher reperfusion injury and also a higher immune response in grafts exposed to additional ischemia. Whether HOPE is also effective, for example, in allogeneic donated after cardiac death livers or in allogeneic liver grafts with long cold ischemia requires further investigation.

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